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Hailiang Sun

Mississippi State University

Fred L. Cunningham

USDA/APHIS/WS National Wildlife Research Center Mississippi Field Station, fred.l.cunningham@aphis.usda.gov

Jillian Harris

Mississippi State University

Yifei Xu

Mississippi State University

Li-Ping Long

Mississippi State University

See next page for additional authors

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Authors

Hailiang Sun, Fred L. Cunningham, Jillian Harris, Yifei Xu, Li-Ping Long, Katie Hanson-Dorr, John A. Baroch, Paul Fioranelli, Mark W. Lutman, Tao Li, Kerri Pedersen, Brandon S. Schmit, Jim Cooley, Xiaoxu Lin, Richard G. Jarman, Thomas J. DeLiberto, and Xiu-Feng Wan

Dynamics of virus shedding and antibody responses in influenza A virus-infected feral swine

Hailiang Sun,^{1†} Fred L. Cunningham,^{2†} Jillian Harris,¹ Yifei Xu,¹ Li-Ping Long,¹ Katie Hanson-Dorr,² John A. Baroch,³ Paul Fioranelli,² Mark W. Lutman,⁴ Tao Li,⁵ Kerri Pedersen,⁴ Brandon S. Schmit,³ Jim Cooley,⁶ Xiaoxu Lin,⁵ Richard G. Jarman,⁵ Thomas J. DeLiberto³ and Xiu-Feng Wan¹

Correspondence
Xiu-Feng Wan
wan@cvm.msstate.edu

¹Department of Basic Sciences, College of Veterinary Medicine, Mississippi State University, Mississippi State, MI, USA

²Mississippi Field Station, National Wildlife Research Center, Wildlife Services, Animal and Plant Health Inspection Service, US Department of Agriculture, Mississippi State, MI, USA

³National Wildlife Research Center, Wildlife Services, Animal and Plant Health Inspection Service, US Department of Agriculture, Fort Collins, CO, USA

⁴US Department of Agriculture, Animal and Plant Health Inspection Service, Wildlife Services, Fort Collins, CO, USA

⁵Viral Diseases Branch, Walter Reed Army Institute of Research, Silver Spring, MD, USA

⁶Department of Pathobiology and Population Medicine, College of Veterinary Medicine, Mississippi State University, Mississippi State, MI, USA

Given their free-ranging habits, feral swine could serve as reservoirs or spatially dynamic 'mixing vessels' for influenza A virus (IAV). To better understand virus shedding patterns and antibody response dynamics in the context of IAV surveillance amongst feral swine, we used IAV of feral swine origin to perform infection experiments. The virus was highly infectious and transmissible in feral swine, and virus shedding patterns and antibody response dynamics were similar to those in domestic swine. In the virus-inoculated and sentinel groups, virus shedding lasted ≤ 6 and ≤ 9 days, respectively. Antibody titres in inoculated swine peaked at 1 : 840 on day 11 post-inoculation (p.i.), remained there until 21 days p.i. and dropped to $< 1 : 220$ at 42 days p.i. Genomic sequencing identified changes in wildtype (WT) viruses and isolates from sentinel swine, most notably an amino acid divergence in nucleoprotein position 473. Using data from cell culture as a benchmark, sensitivity and specificity of a matrix gene-based quantitative reverse transcription-PCR method using nasal swab samples for detection of IAV in feral swine were 78.9 and 78.1 %, respectively. Using data from haemagglutination inhibition assays as a benchmark, sensitivity and specificity of an ELISA for detection of IAV-specific antibody were 95.4 and 95.0 %, respectively. Serological surveillance from 2009 to 2014 showed that ~ 7.58 % of feral swine in the USA were positive for IAV. Our findings confirm the susceptibility of IAV infection and the high transmission ability of IAV amongst feral swine, and also suggest the need for continued surveillance of IAVs in feral swine populations.

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INTRODUCTION

Influenza viruses (family *Orthomyxoviridae*) are classified into types A, B and C (Alexander & Brown, 2000; Mahy, 1997). Influenza A virus (IAV), an enveloped RNA virus,

contains eight negative-sense ssRNA genome segments. The IAVs can cause seasonal epidemics, affecting one or many countries, as well as pandemics. IAVs have been recovered from at least 105 wild bird species of 26 different families (Olsen *et al.*, 2006a), and species living in wetland and aquatic environments (e.g. *Anseriformes* and *Charadriiformes* spp.) constitute the major natural IAV reservoir (Webster *et al.*, 1992). However, in addition to circulating amongst avian species, IAVs also circulate amongst a wide

†These authors contributed equally to this work.

Two supplementary tables are available with the online Supplementary Material.

spectrum of other host species, including humans, swine, equines, canines and marine mammals (Keawcharoen *et al.*, 2004; Peiris *et al.*, 2007; Sun *et al.*, 2011; Webster *et al.*, 1992).

Amongst the natural hosts of IAVs, swine have been shown to be susceptible to many IAV subtypes (Kida *et al.*, 1994). In domestic swine, IAVs can cause respiratory diseases characterized by fever, lethargy, sneezing, coughing, difficulty breathing and decreased appetite, which usually lead to weight loss. For the past decade, IAV subtypes H1N1, H1N2 and H3N2 have been the predominant strains circulating amongst the domestic swine population in the USA (Vincent *et al.*, 2008). Antigenic characterization revealed that the circulating H1N1 IAVs formed four genetic clusters: swH1 α (classic H1N1), swH1 β (reassortant H1N1-like), swH1 γ (H1N2-like) and swH1 δ (human-like H1). Viruses within cluster swH1 δ can be further classified into two subclusters: swH1 δ 1 (human-like H1N2) and swH1 δ 2 (human-like H1N1) (Vincent *et al.*, 2006, 2009). The 2009 pandemic influenza A(H1N1)pdm09 virus is a classic subtype H1N1-origin swine virus, but it differs genetically from the four genetic clusters identified from the USA (Lorusso *et al.*, 2011). Antigenic characterization showed variations amongst viruses in the subtype H1N1 clusters (Lorusso *et al.*, 2011).

Similar to H1 IAVs, the H3N2 subtypes in the US swine population are also genetically and antigenically diverse. Four genetic clusters of H3N2 subtype IAVs (clusters I–IV) have been identified (Hause *et al.*, 2010; Olsen *et al.*, 2006b; Richt *et al.*, 2003). Cluster IV, which has become predominant amongst the US swine population, has further evolved into two antigenic clusters: H3N2- α and H3N2- β (Feng *et al.*, 2014). Many of these H3N2 genetic clusters are currently co-circulating in swine populations and frequent reassortments of these IAVs have occurred. In 2011, a predominant H3N2 genotype containing a matrix gene from influenza A(H1N1)pdm09 virus led to the emergence of an H3N2 variant virus that caused disease in humans (Bowman *et al.*, 2012; Nelson *et al.*, 2012; Shu *et al.*, 2012); this variant IAV is antigenically similar to H3N2- β viruses (Feng *et al.*, 2014).

In addition to the prevalent H1 and H3 IAVs, other haemagglutinin subtype viruses, such as H1N1, H4N6, H5N1, H6N6 and H9N2, have been transiently detected in swine (Choi *et al.*, 2005; Guan *et al.*, 1996; Olsen, 2002; Peiris *et al.*, 2001; Zhang *et al.*, 2011). As swine have the avian-like NeuAc-2,3- α -Gal receptors and the human-like NeuAc-2,6- α -Gal receptors in their respiratory tracts, they have been proposed as a 'mixing vessel' for the generation of IAV reassortants (Scholtissek, 1994).

In the USA, there are ~5 million feral swine across >40 states and the number is increasing (Bevins *et al.*, 2014; Fogarty, 2007). Contacts between feral and domestic swine provide the opportunities for bi-directional transmission for pathogens such as IAVs (Wyckoff *et al.*, 2009). Also, because feral swine and wild birds are

sympatric throughout their ranges, direct contact (e.g. feral swine scavenging and predation on wild birds) and indirect contact (e.g. use of common resources like water and forage) provide the opportunity for virus transmission amongst these species (Bevins *et al.*, 2014). The free-ranging nature of feral swine and their direct and indirect interactions with other IAV hosts position them as ideal, spatially dynamic 'mixing vessels'. Previous studies have recovered subtype H3N2 and influenza A(H1N1)pdm09 viruses from feral swine (Clavijo *et al.*, 2012). Results from serological surveillance during October 2011–September 2012 documented that 9.15 % of serum samples from feral swine in 31 states were positive for IAV exposure, of which >60 % were positive for H3 subtype viruses (Feng *et al.*, 2014). These findings indicate that IAVs are widely present in feral swine, and IAVs circulating amongst feral swine are antigenically and genetically similar to those circulating in domestic swine.

Surveillance for IAV infection is more challenging in feral swine than in domestic swine because multiple haemagglutinin subtypes can be present in feral swine and little is known about IAV infection dynamics in these animals. Current methods for IAV diagnosis and surveillance in feral swine were adapted from protocols designed for use with domestic swine or poultry. To improve these methods and to evaluate protocols for IAV diagnosis and surveillance in feral swine, knowledge of virus shedding patterns and antibody response dynamics is needed. Furthermore, it is known that feral swine can acquire IAV from domestic swine (Bevins *et al.*, 2014; Fogarty, 2007), but it is not clear whether IAVs can be easily transmitted amongst feral swine.

To better understand IAV shedding patterns, antibody response dynamics and the potential for IAV transmission amongst feral swine, we performed infection and reinfection experiments using a subtype H3N2 IAV of feral swine origin. Other goals were to investigate whether feral swine with low IAV antibody titres could infect and shed homologous viruses, and to evaluate the protocol for influenza diagnosis and surveillance in feral swine.

RESULTS

H3N2 IAV is highly infectious and transmissible in feral swine

We determined virus shedding patterns and antibody response dynamics in 12 feral swine by performing infection experiments with a subtype H3N2 IAV of feral swine origin that was highly infectious and transmissible amongst these animals. The swine were divided into treatment ($n=8$) and sentinel ($n=4$) groups, and housed at one or two animals per pen (a total of six adjoining pens for the treatment group and three for the sentinel group); pens for treatment and sentinel groups were 1.2 m apart (Fig. 1). Swine in the treatment group began shedding virus 1 day post-inoculation

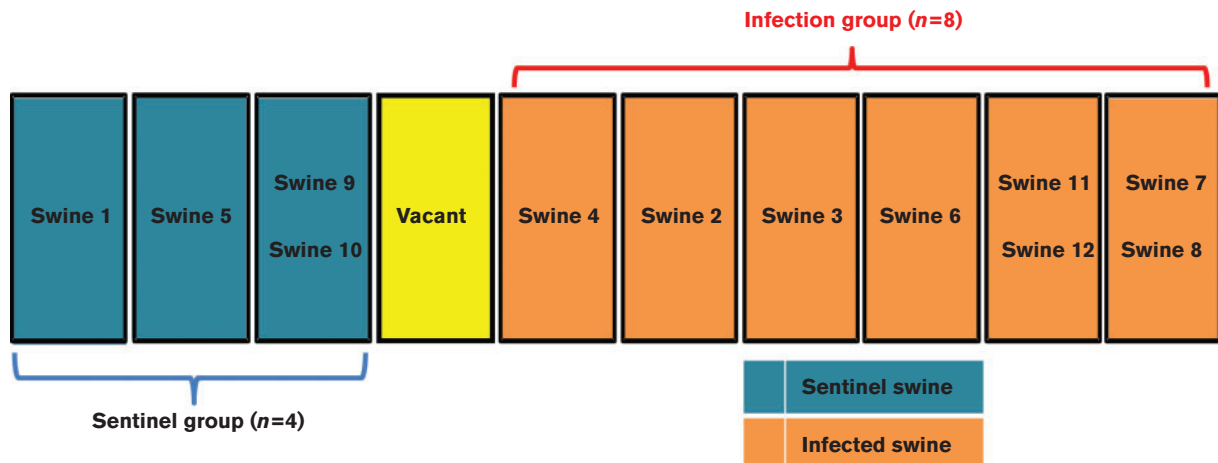


Fig. 1. Physical layout of pens housing swine in a study of the virus shedding patterns and antibody response dynamics during IAV infection in feral swine. An empty pen was located between six pens housing a total of eight swine in the treatment group and three pens housing a total of four swine in the sentinel group. Swine in the treatment group were intranasally inoculated twice (days 0 and 103) with 10^6 TCID₅₀ influenza A/swine/Texas/A01104013/2012(H3N2) virus; swine in the sentinel group were intranasally inoculated once (day 103) with 10^6 TCID₅₀ influenza A/swine/Texas/A01104013/2012(H3N2) virus.

(p.i.) and continued to shed virus until 6 days p.i. Virus titres in nasal wash and nasal swab samples from the treatment group ranged from $10^{0.575}$ to $10^{1.878}$ TCID₅₀ ml⁻¹ and peaked at 5 days p.i. In the four sentinel swine, virus shedding was detectable 1 day post-exposure (p.e.) to the IAV-inoculated swine. Virus titres in sentinel swine peaked at 8 days p.e. (Fig. 2) and shedding was sustained for up to 9 days (mean titres $10^{1.532}$ – $10^{4.673}$ TCID₅₀ ml⁻¹). No obvious clinical signs of infection (e.g. cough, fever, weight loss) were observed in the inoculated or sentinel swine. To investigate whether feral swine with low IAV antibody titres could infect and shed homologous viruses, we reinoculated swine in the treatment group and inoculated swine in the sentinel group with subtype H3N2 IAV on day 103 p.i. However, the antibody in the treatment swine quickly rose and we were unable to determine virus titres in samples from the treatment group.

Antibody response dynamics in feral swine after inoculation with H3N2 IAV

Feral swine in the treatment group seroconverted at 8 days p.i.; the mean haemagglutination inhibition (HI) titre was 1 : 450 (Fig. 3). The mean HI titres peaked at 1 : 840 at 11 days p.i. and remained at that level until 21 days p.i. before gradually declining to < 1 : 220 at 42 days p.i.; titres then continued to decline to 1 : 130 at 93 days p.i. Before swine in the treatment group were reinoculated with H3N2 IAV at 103 days p.i., the mean HI titre was 1 : 120.

Feral swine in the sentinel group seroconverted (mean HI titre 1 : 60) at 11 days p.e.; HI titres in these swine peaked at 1 : 960 at 21 days p.e. and gradually declined to 1 : 373

at 42 days p.e. Before swine in the sentinel group were inoculated with H3N2 IAV at 103 days p.e., the mean HI titre was 1 : 213.

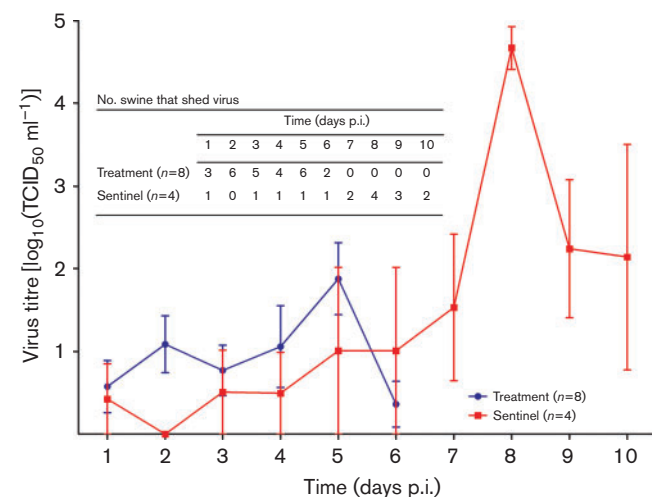


Fig. 2. Mean titres of influenza viruses recovered from nasal wash or nasal swab samples of feral swine following nasal inoculation of influenza virus. Swine in the infection group were inoculated with 10^6 TCID₅₀ influenza A/swine/Texas/A01104013/2012(H3N2) virus (in 1 ml volume). Swine in the sentinel group were inoculated with 1 ml PBS. Virus titres were measured in nasal wash or swab fluids collected on indicated days following titration in Madin-Darby canine kidney (MDCK) cells; ending titres are expressed as mean \pm SD. The inset table shows the number of swine that shed virus on the various days after virus inoculation or exposure.

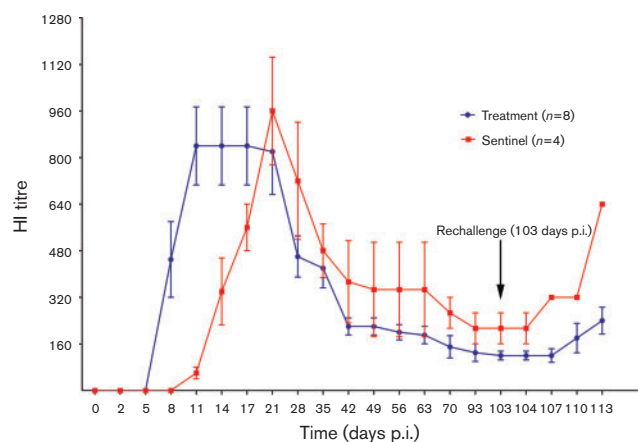


Fig. 3. Mean HI titres of IAVs in serum samples collected from feral swine following nasal inoculation of influenza virus. Swine in the infection group were inoculated twice (days 0 and 103) with 10^6 TCID₅₀ influenza A/swine/Texas/A01104013/2012(H3N2) virus (in 1 ml volume). Swine in the sentinel group were inoculated once with 1 ml PBS (day 0) and once (day 103) with 10^6 TCID₅₀ influenza A/swine/Texas/A01104013/2012(H3N2) virus (in 1 ml volume). HI titres were measured in serum samples obtained on indicated days following inoculation. HI assays were conducted by using 0.5% turkey red blood cells. Titres are expressed as mean \pm SD from the results in eight swine in the treatment group or four swine in sentinel group.

After swine in the treatment group were reinoculated and the sentinel group was inoculated with IAV, the antibody titre increased in both groups of swine. At 113 days p.i. (10 days after reinoculation), HI titres in the treatment group swine increased to 1 : 240, whereas titres in the sentinel swine group increased to 1 : 640 (Fig. 3).

During 21–113 days p.i./p.e., antibody titres in sentinel swine were significantly higher ($P < 0.001$) than those in inoculated swine.

Amino acid polymorphisms in IAVs recovered from experimentally infected feral swine

To explore the mechanism for the discrepancy in antibody levels between sentinel and treated swine, we recovered viruses from the sentinel swine and compared them with the WT virus that was used to inoculate swine in the treatment group. The growth kinetics study demonstrated that, compared with virus isolates from the sentinel swine, WT virus replicated better in Madin–Darby canine kidney (MDCK) cells (Fig. 4). Titres of WT virus were 0.5- to 1.5-fold higher ($P < 0.05$) than titres for sentinel swine test isolate P10D8 at 24, 48 and 72 h p.i., and for sentinel swine test isolate P5D8 at 24 h p.i. Further sequencing of these isolates identified amino acid and nucleic acid polymorphisms in PB2, PB1, PA, HA, NP, NA, M1, and NS1, (Tables 1, S1 and S2, available in the online Supplementary Material). Overall, these polymorphisms suggested that the

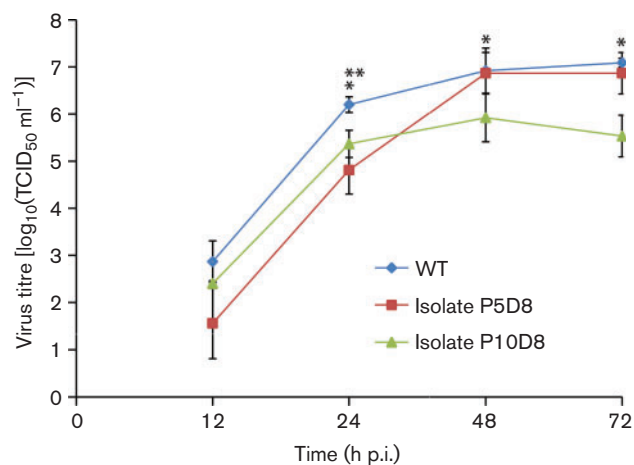


Fig. 4. Growth kinetics of influenza virus isolates and WT virus characterized in MDCK cells. MDCK cells were infected with individual viruses at m.o.i. 0.001. Virus titres were measured in MDCK cells; ending titres are expressed as mean \pm SD from three independent experiments. *WT virus is significantly higher than that of isolate P10D8 ($P < 0.05$); **WT virus is significantly higher than that of isolate P5D8 ($P < 0.05$).

original cell-adapted virus inoculum adapted within the infected swine and that this adaptation may underlie the increase in HI titre levels.

Sensitivity of nasal swab specimens for diagnosing IAV infection in feral swine

To assess the value of a matrix gene-based quantitative reverse transcription (qRT)-PCR method using nasal swab samples for determining IAV infection, we compared qRT-PCR and TCID₅₀ results for 141 swab samples collected 1–14 days p.i. (Tables 2 and 3). The qRT-PCR results were positive for 55 samples and cell cultures were positive for 41 samples; 32 of the samples had positive results from both the qRT-PCR and cell culture methods. The sensitivity and specificity of the qRT-PCR method using nasal swab samples for diagnosis of IAV infection in feral swine was 78.90 and 78.05%, respectively.

Evaluation of current protocols for using an AI Multi-Screen Ab Test kit to detect IAV in feral swine

To evaluate current protocols for IAV surveillance in the feral swine population, we used an AI Multi-Screen Ab Test kit (see Methods) to determine the receiver operating characteristic curve. Results showed that when the threshold of the sample-to-negative control (S/N) ratio increased, the sensitivity of the AI Multi-Screen Ab Test kit also increased, but specificity decreased (Fig. 5). When the S/N threshold was 0.50, sensitivity and specificity of the AI Multi-Screen Ab assay were 81.44 and 100%, respectively. When the S/N threshold was increased to

Table 1. Amino acid polymorphisms amongst sequences of WT virus used for inoculation and amongst virus isolates recovered from sentinel swine in a study of the dynamics of virus shedding and antibody responses during IAV infection in feral swine

Gene	Position*	Amino acid† (by virus‡)			
		P1D8	P5D8	P10D8	WT
HA	443	L/H (137/65)	L	L	L
NA	346	G	G/S (279/211)	G	G
PB1	59	T	T	T/K (2041/575)	T
NP	473	S	S	S/G (2223/232)	S/G (459/275)
NS1	157	V	V	I/V (803/6400)	I/V (1747/1605)

*Numbering of the residues was from the first amino acid in the methionine start site of each gene of the influenza viruses.

†Numbers in parentheses represent number of supporting reads.

‡Isolates P1D8, P5D8 and P10D8 were isolated from sentinel swine numbers 1, 5 and 10, respectively, 8 days after swine in the treatment group were infected. WT virus was influenza strain A/swine/Texas/A01104013/2012(H3N2) that was used to inoculate swine in the treatment group.

0.681 (i.e. the value currently used by the US Department of Agriculture as the standard for testing feral swine samples for IAV exposure), sensitivity and specificity of the AI MultiS-Screen Ab assay increased to 95.36 and 95.00 %, respectively. If specificity and sensitivity were combined, an S/N value of 0.681 would be reasonable for the diagnosis of IAV in feral swine. By applying the S/N threshold of 0.681, we determined that 7.58 % (585) of 7714 serum samples collected from feral swine during 2009–2014 were positive for IAV (US Department of Agriculture, unpublished data); this finding confirms the prevalence of IAV infection in feral swine. Our results

also demonstrated that the 0.681 threshold currently used in field surveillance programs is effective in identifying IAV-positive serum samples from feral swine.

DISCUSSION

Under laboratory conditions, enzootic swine-origin subtype H1N1, H3N2 and H1N2 IAVs have been shown to replicate in the respiratory tracts of domestic swine, and virus shedding lasts for 5–6 days (De Vleeschauwer *et al.*, 2009a; Van Reeth *et al.*, 2009). However, infection with avian-origin subtype H1N1, H4N1, H5N1 and H7N1 IAVs results

Table 2. Results of qRT-PCR of nasal swab samples from feral swine after inoculation with influenza A/swine/Texas/A01104013/2012(H3N2) virus and from feral swine after exposure to infected swine

NC, no samples were collected.

Group, swine no.*	C _t value (day p.i./p.e.)														
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Inoculation															
2	39.5	29.2	22.1	26.4	22.3	27.7	28.8	34.1	38.2	39.5	0	0	0	0	0
3	0	33.5	NC	24.4	NC	30.2	NC	37.1	NC	0	NC	0	NC	0	NC
4	0	34.1	29.2	26.0	28.3	27.2	28.8	33.8	36.8	39.2	0	33.3	0	37.1	34.1
6	0	35.5	NC	30.2	NC	26.9	NC	32.7	NC	0	NC	0	NC	0	NC
7	0	36.0	NC	30.0	NC	28.3	NC	40.0	NC	0	NC	0	NC	0	NC
8	0	0	NC	31.1	NC	32.8	NC	38.6	NC	0	NC	0	NC	0	NC
11	0	36.9	30.4	27.7	28.6	28.5	33.9	35.6	40.6	0	0	0	0	0	0
12	0	34.3	29.2	28.4	28.4	29.0	31.1	34.4	36.6	0	0	0	0	0	0
Sentinel															
1	0	0	0	0	0	39.5	40.0	29.8	26.0	26.2	30.3	35.5	37.2	0	0
5	0	0	0	0	33.3	24.1	26.3	25.9	23.9	28.1	31.8	34.9	35.5	39.1	37.9
9	0	0	NC	0	NC	37.8	NC	34.4	NC	32.8	NC	39.5	NC	0	NC
10	0	40.9	NC	0	NC	0	NC	36.8	NC	33.1	NC	32.9	NC	33.5	NC

*A feral swine index was used to randomly assign swine to the treatment or sentinel group. Swine in both groups were housed in pens in the same barn (Fig. 1). The following swine were housed together: swine 7 and 8, swine 9 and 10, and swine 11 and 12.

Table 3. Virus titration results for nasal wash and nasal swab samples from feral swine after inoculation with influenza A/swine/Texas/A01104013/2012(H3N2) virus and from feral swine after exposure to infected swine

Group, swine no.*	Titre [$\log_{10}(\text{TCID}_{50} \text{ ml}^{-1})$]† (day p.i./p.e.)													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Inoculation														
2	2.199	2.199	0.699	3.699	2.199	2.199	0	0	0	0	0	0	0	0
3	1.699	2.366	0.699	0	3.032	0	0	0	0	0	0	0	0	0
4	0	0.699	0.699	0.699	3.199	0.699	0	0	0	0	0	0	0	0
6	0.699	0.699	0	0	2.199	0	0	0	0	0	0	0	0	0
7	0	0.699	0	0	2.366	0	0	0	0	0	0	0	0	0
8	0	0	0	2.366	0	0	0	0	0	0	0	0	0	0
11	0	0	2.366	0	0	0	0	0	0	0	0	0	0	0
12	0	2.032	1.699	1.699	2.032	0	0	0	0	0	0	0	0	0
Sentinel														
1	0	0	0	0	0	0	2.93	5.199	4.032	2.866	0	0	0	0
5	1.699	0	2.032	1.98	4.032	4.032	3.199	5.032	2.468	0	0	0	0	0
9	0	0	0	0	0	0	0	4.199	0	0	0	0	0	0
10	0	0	0	0	0	0	0	4.262	2.468	5.699	0	0	0	0

*A feral swine index was used to randomly assign swine to the treatment group or the sentinel group. Swine in the treatment group and the sentinel group were housed in pens in the same barn (Fig. 1). The following swine were housed together: swine 7 and 8, swine 9 and 10, and swine 11 and 12.
 †Virus titres were measured in MDCK cells; the titres in italics were for nasal wash samples, and the others for nasal swab samples.

in virus shedding for 3–6 days (De Vleeschauwer *et al.*, 2009b). Furthermore, influenza A(H1N1)pdm09 virus infection in domestic swine can lead to virus shedding for up to 10 days (Bragstad *et al.*, 2013). Thus, the duration of virus shedding in IAV-infected domestic swine depends on the virus subtype and strain. In this study, we showed that feral swine infected with the H3N2 IAV of feral swine origin can shed virus for up to 10 days.

After infection, no obvious clinical signs were observed in feral swine of either group; domestic swine infected with IAVs show only mild respiratory distress or, sometimes, no clinical signs (Bragstad *et al.*, 2013; Ferrari *et al.*, 2010). The virus titration results suggested low shedding in both treatment and sentinel swine. However, the high HI titres suggested that both groups were successfully infected with H3N2 virus. It should be noted that this H3N2 IAV replicated poorly in MDCK cells and thus virus titration in MDCK cells could generate biases in the virus shedding data.

Results showed that virus titres in the sentinel group were higher than those in the treatment group. This finding is consistent with results from another swine experiment with influenza strain A/swine/Flanders/1/98(H3N2) (De Vleeschauwer *et al.*, 2009b). In that study, nasal shedding in inoculated pigs peaked at 1 day p.i. [titre of $\sim 10^5 \text{ EID}_{50}$ (100 mg nasal secretions) $^{-1}$, where EID_{50} is the 50% egg infectious dose] and shedding continued for ~ 7 days; however, nasal shedding in contact pigs peaked at 6 days p.e. [titre of 10^6 EID_{50} (100 mg nasal secretions) $^{-1}$]. Other reports showed evidence of higher replication capability

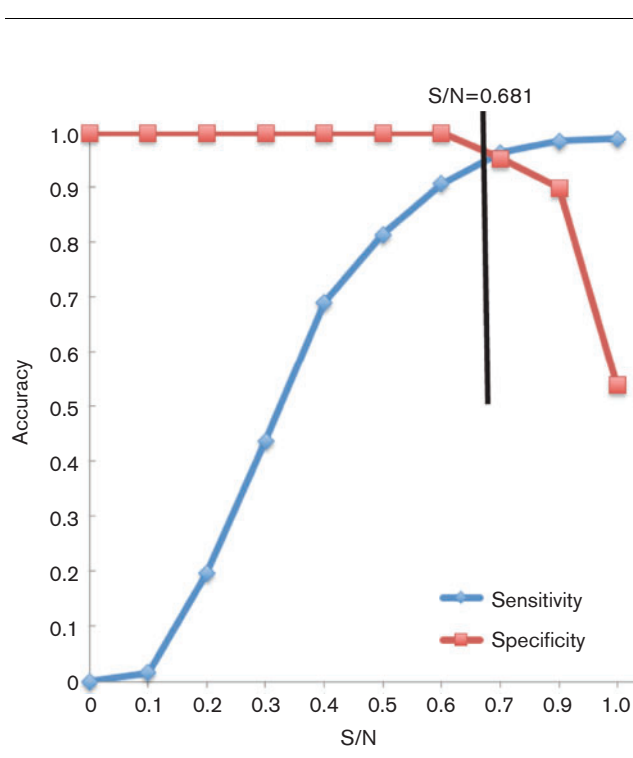


Fig. 5. Receiver operating characteristic curves showing sensitivity and specificity of ELISAs for detection of IAV in experimentally infected feral swine and in sentinel swine exposed to infected swine. The threshold of 0.681 for the S/N was used by the US Department of Agriculture and this cut-off is indicated by a black straight line.

and/or pathogenesis by IAV after passage in a host, suggesting possible adaptation of the viruses to host species (Mase *et al.*, 2006; Wei *et al.*, 2014). Thus, it is likely that before being transmitted from treatment group swine to sentinel swine, the WT viruses acquired improved replication efficiency and infection capability during passage in the treatment group swine. Of note, the viruses inoculated into the treatment group swine had been passaged twice in MDCK cells. Furthermore, the data obtained from genomic sequencing identified a few changes between the isolates recovered from the sentinel swine and the WT viruses inoculated into the treatment swine: changes were noted in non-protein-coding sequences, and in amino acid and nucleotide sequences in the protein-coding sequences (Tables 1, S1 and S2). Notably, there was amino acid divergence in position 473 in the nucleoprotein. Amino acid at position 473 (in the peptide of 473–481) in the nucleoprotein was relatively conserved in subtype H1N1 and H3N2 IAVs, and was reported to stimulate the host to produce IFN- γ (Wahl *et al.*, 2009). These findings are of interest because the non-coding regions and some coding regions of genome segments affect viral RNA synthesis and packing (Liang *et al.*, 2005; Muster *et al.*, 1991; Ng *et al.*, 2008; Sun *et al.*, 2015; Watanabe *et al.*, 2003; Zheng *et al.*, 1996). Further experiments are needed to validate the roles of sequence changes in the discrepancy seen in patterns of virus shedding and antibody response dynamics between the swine in the treatment and sentinel groups.

It is likely that seroconversion of sentinel swine occurred after they were exposed to contaminated fomites or aerosolized virus disseminated through the ventilation system. This supposition is supported by the fact that one sentinel swine began shedding virus at 1 day p.e., even though virus detection by qRT-PCR was negative for this swine until 5 days p.e. (Table 2). Although it is possible that infectious aerosols (e.g. those created during pen washing) or contact with infectious fomites could have led to virus transmission amongst swine (Allerson *et al.*, 2013; Tellier, 2006), the sentinel and treatment groups were separated by 1.2 m, and our study protocol included procedures to avoid the unintended transmission of virus as a result of routine cleaning and feeding (e.g. sentinel group pens were cleaned before treatment group pens and the sentinel group was fed before the treatment group). Regardless, the findings in this study demonstrate that subtype H3N2 IAV can be easily transmitted amongst feral swine.

Isolation of enzootic subtype H3N2 and H1N1 IAVs from feral swine suggested that there have been multiple introductions of IAVs from domestic swine to feral swine (Bevins *et al.*, 2014; Fogarty, 2007). It would be of interest to know whether these introduced H3N2 IAVs can be maintained in the feral swine population. In domestic swine, subtype H3N2 IAV can persist in the population through chronic infection of swine or periodic reintroduction of the virus (Kyriakis *et al.*, 2013; Wallace, 1979). As feral swine can live ~ 8 years (versus < 1 year for most domestic swine) and are free-ranging (unlike domestic

swine), they have more opportunities to be exposed to other feral swine, domestic swine and ponds contaminated by avian influenza-infected wild birds, including migratory waterfowl. Our findings suggest that H3N2 virus of feral swine origin can be transmitted easily amongst feral swine. Thus, feral swine could facilitate antigenic drift or shift, which could lead to the generation of novel IAVs, further complicating our ability to elucidate the complex ecology of IAVs.

Swine in the treatment group seroconverted (HI titres $\geq 1:40$) at 8 days p.i. and swine in the sentinel group seroconverted 3 days later. These results were similar to those in a study of domestic swine experimentally infected with H1 IAV, in which seroconversion occurred at 7–8 days p.i. (Lee *et al.*, 1995; Panyasing *et al.*, 2013). In addition, another study showed that antibody titres in IAV-inoculated domestic swine were lower than those in domestic swine infected through contact transmission (De Vleeschauwer *et al.*, 2009b). Our finding that mean HI titres at different time points were higher in the sentinel group than in the treatment group were also consistent with findings in these previous studies. Unfortunately, we were unable to detect viruses in nasal wash samples from swine that were reinoculated with H3N2 IAV at 103 days p.i. because of a spike in HI antibody titre levels.

Although next-generation sequencing detected a few amino acid polymorphisms across the genomes of WT virus and virus isolates recovered from sentinel swine, it is still not clear whether these polymorphisms led to the discrepancy in antibody levels between sentinel and treated swine. Further experiments using IAV reassortants with a specific mutation are needed to identify any specific polymorphisms responsible for such a difference.

A previous study in feral swine documented a very low rate of virus recovery: amongst 1983 nasal swab samples from feral swine, nine were matrix gene qRT-PCR-positive and only one IAV strain was isolated (Feng *et al.*, 2014). Our study demonstrated that nasal swab samples from feral swine provide adequate virus for the detection of IAV. The low rates of IAV detection and isolation in the previous study (Feng *et al.*, 2014) were probably due to the short duration of virus shedding. Nasal swab samples from younger, immunologically naive feral swine would more likely lead to a higher qRT-PCR detection and virus isolation success rate.

Comparison of virus titres for swine with only nasal swab samples and those with alternating nasal wash and nasal swab samples demonstrated that the virus titres in the former group were slightly higher than those in the latter group (Table 3). It is interesting that, once virus shedding was detected, the swine with only swab samples had consistent shedding patterns, whereas those with alternating nasal wash and nasal swab samples did not. This might have occurred because nasal washes likely cleared some viral particles from the nasal cavity.

Serological analyses demonstrated that ELISAs are less sensitive than HI assays for the detection of IAV in feral swine. However, by optimizing the S/N threshold, both the sensitivity and specificity of ELISAs can be improved to >95 % with a S/N threshold of 0.681. With this threshold, the serological surveillance from 2009 to 2014 showed that ~7.58 % of feral swine in the USA were positive for IAV, confirming the prevalence of IAV infection in feral swine. These findings suggest the need for continued surveillance to monitor the distribution, and the genomic and antigenic diversities of IAVs in feral swine populations.

METHODS

Virus and cells. We used influenza A/swine/Texas/A01104013/2012(H3N2) virus, a WT strain of feral swine origin (Feng *et al.*, 2014), to infect feral swine. MDCK cells (American Type Culture Collection) were maintained at 37 °C with 5 % CO₂ in Dulbecco's modified Eagle's medium (Gibco-BRL) supplemented with 10 % FBS (Atlanta Biologicals) and penicillin/streptomycin (Gibco-BRL). Virus amplification was performed in MDCK cells at 37 °C with 5 % CO₂ in Opti-MEM I Reduced Serum Medium (Life Technologies) supplemented with TPCK (L-1-tosylamido-2-phenylethyl chloromethyl ketone)-treated trypsin (1.5 µg ml⁻¹).

RNA extraction, PCR, qRT-PCR and genomic sequencing. Viral RNA was extracted by using an RNeasy Plus Mini kit (Qiagen). The full-length cDNA for eight influenza gene segments was amplified by using SuperScript One-Step RT-PCR (Invitrogen) with previously described primers (Hoffmann *et al.*, 2001; Zhou *et al.*, 2009). To address the possibility of mutations changing the growth phenotype of viruses, the full genome of isolates from sentinel swine and WT virus used for inoculation were amplified by using a method described previously (Zhou *et al.*, 2009). Amplified viral DNA products were quantified by using a High Sensitivity DNA kit on an Agilent 2100 Bioanalyzer (Agilent Technologies). An equal amount of each sample was used with an Illumina Nextera DNA Sample Preparation kit (Illumina) to prepare a sequencing library. Library samples were further quantified, normalized and then pooled together. Pooled library samples were sequenced by using a MiSeq Reagent kit version 2 (500 cycles) on a MiSeq sequencer (Illumina) according to the sequencing protocol suggested by the manufacturer.

Feral swine. A total of 12 feral swine (body weight 16–22 kg) were trapped in a rural area of Starkville, MS, USA, by using corral traps constructed of 4.9 × 1.5 m utility panels with 10.2 cm² mesh and steel T-posts. Animals were transported in an air-conditioned, enclosed trailer to the Wildlife Services, National Wildlife Research Center, Mississippi Field Station in Mississippi State, MS, USA. The captured swine were quarantined for 1 week, and tested for exposure to brucellosis, pseudorabies and IAV by ELISA; all test results were negative. The swine were then housed in 1.2 × 2.7 m pens with solid concrete floors in an enclosed, air-conditioned building (Fig. 1). Once a day, the swine were fed a 16 % crude protein commercial swine ration equal to 4 % of their body weight.

Animal experiments. The 12 feral swine were each assigned to one of two groups: a treatment group (*n*=8) or a sentinel group (*n*=4). Animals in the treatment group were housed in six adjoining pens (one or two swine per pen); swine in the sentinel group were housed in three adjoining pens (one or two swine per pen), separated from those in the treatment group by 1.2 m (Fig. 1).

Prior to virus inoculation and sampling, animals were anaesthetized by using a syringe pole to inject TKX (4.4 mg Telazol kg⁻¹, 2.2 mg

ketamine kg⁻¹ and 2.2 mg xylazine kg⁻¹) (Ko *et al.*, 1993) at 0.044 ml (kg body weight)⁻¹ behind the ear, in the loin or in the ham. Animals in the treatment group were intranasally inoculated with 10⁶ TCID₅₀ influenza A/swine/Texas/A01104013/2012(H3N2) in 1 ml volume (Fig. 1). To investigate whether feral swine with low IAV antibody titres could infect and shed homologous viruses, we reinoculated swine in the treatment group at 103 days p.i. and we intranasally inoculated swine in the sentinel group with 10⁶ TCID₅₀ (in 1 ml volume) of the same virus. We originally planned to administer these inoculations at 90 days p.i., but at that time, the US government had shut down all non-essential government work. Thus, we had to postpone the reinfection experiments until 103 days p.i., when we were permitted to continue our experimental activities.

Nasal swab samples were collected from half of the swine (four treatment and two sentinel swine) daily from 1 to 14 days p.i. and from 104 to 113 days p.i., and stored in 15 ml tubes with 2 ml PBS. In the remaining swine (four treatment and two sentinel swine), nasal swab or nasal wash samples were collected on alternating days from 1 to 14 days p.i. and from 104 to 113 days p.i. Nasal washes were performed in both nostrils by using 2 ml PBS. qRT-PCR was used to determine virus loads in nasal swab samples, and viruses in both nasal wash and nasal swab samples were titrated by TCID₅₀. Blood samples (5 ml) were obtained from swine for serological assays at 0, 2, 5, 8, 11, 14, 17, 21, 28, 35, 42, 49, 56, 63, 70, 93, 103, 104, 107, 110, and 113 days p.i.

Two swine per day (one treatment and one sentinel) were euthanized on 104–106 days p.i. and necropsies were performed. Euthanasia was performed by administration of barbiturates solution [1 ml (4.5 kg body weight)⁻¹] in fully anaesthetized swine [0.044 ml TKX (kg body weight)⁻¹]. The remaining six swine were euthanized at 113 days p.i. and necropsies were performed.

Swine were monitored daily for subjective signs of influenza infection (e.g. lethargy, nasal discharge, coughing and dyspnoea) and objective signs (e.g. body temperature) until 14 days p.i. Body weight was measured weekly.

Serological assays. To determine the antibody response dynamics in feral swine after IAV infection, we conducted a HI assay, using 0.5 % turkey red blood cells, as described previously (Sun *et al.*, 2013). ELISAs were performed by using an AI MultiS-Screen Ab Test kit (IDEXX).

Growth kinetics of viruses in MDCK cells. To characterize the growth kinetics of influenza strain A/swine/Texas/A01104013/2012(H3N2), we inoculated MDCK cells with the virus at m.o.i. 0.001. After being incubated for 1 h at 37 °C, the inoculum was removed and the cells were washed three times in PBS. The cells were then incubated (37 °C in 5 % CO₂) in Opti-MEM I (Gibco) containing TPCK-treated trypsin (1.5 µg ml⁻¹). At 12, 24, 48 and 72 h p.i., supernatant was collected from three of the 12 wells (1 ml per well) and then titrated, by TCID₅₀, in MDCK cells. TCID₅₀ was calculated by using the Reed–Muench method (Reed & Muench, 1938).

Data analyses. Student's *t*-test was used to determine whether serological responses in the treatment group differed from responses in sentinel swine. Specificity and sensitivity of ELISA kits were calculated. Results from HI assays were viewed as true positive and true negative. We similarly measured the specificity and sensitivity of the nasal swab sample-based qRT-PCR method for influenza diagnosis. Cell culture results (TCID₅₀) for nasal swab samples were used as true positive and true negative. The data from the growth curve of the WT virus and the isolates recovered from the sentinel swine were analysed by using a one-way repeated-measure ANOVA followed by Turkey and Duncan's multiple comparison test.

The MiSeq sequence reads were matched to the reference genome by using the Bowtie2 alignment program (Langmead & Salzberg, 2012).

Single nucleotide polymorphisms were analysed by using VarScan version 2.3.6 (Koboldt *et al.*, 2012). A minimum of 10-fold mapping coverage and a quality score of 30 were required for a given nucleotide position. A minor variant was determined only if it was supported by at least 20 % of reads at a given position.

Biosafety protocols for laboratory and animal experiments. The laboratory analysis and inoculation trials in feral swine were carried out under Biosafety Level 2 conditions, with investigators wearing appropriate personal protective equipment, in compliance with the Institutional Animal Care and Use Committee of US Department of Agriculture-approved protocols, and Biosafety in Microbiological and Biomedical Laboratories, as well as Risk Group Classification for Infectious Agents.

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